AD_____

Award Number: W81XWH-07-1-0474

TITLE: Replicating Physiological Patterns of Activity with Prosthetic Stimulation.

PRINCIPAL INVESTIGATOR: Shelley Fried, Ph.D.

CONTRACTING ORGANIZATION: Boston VA Research Institute (BVARI)

Boston, MA 02130

REPORT DATE: July 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE *(DD-MM-YYYY)* | 2. REPORT TYPE | 3. DATES COVERED *(From - To)* |
|---|---|---|
| 01-07-2009 | Annual | 11 Jun 2008 – 10 Jun 2009 |

**4. TITLE AND SUBTITLE**
Replicating Physiological Patterns of Activity with Prosthetic Stimulation

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-07-1-0474

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
Shelley Fried, PhD (PI) – principle author of report
Email: shelleyfried@yahoo.com

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Boston VA Research Institute

Boston, MA 02130

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research
And Materiel Command
Fort Detrick, Maryland
21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release, distribution unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

We want to develop more effective methods of neural stimulation in order to improve the clinical outcomes associated with retinal prosthetics. To accomplish this, we are investigating the mechanism(s) by which different types of retinal neurons respond to electric stimulation. Previous studies have shown that ganglion cells, the output cells of the retina, can be activated directly and exclusively with short duration stimulus pulses[1-4]. However, the site of spike initiation in ganglion cells (e.g. the element with the lowest threshold) is not known. Here, we found that the lowest thresholds occurred along the proximal axon, about 40 μm from the soma; this region of low threshold was spatially coextensive with a band of dense sodium channels also centered about 40 μm from the soma. The sodium channel bands formed a homogeneous population for a given type of ganglion cell (e.g. alpha), but the properties of the band were different across different types (e.g. the lengths and locations varied). As expected from the differences in band properties, the size and location of the low threshold regions were also different for different ganglion cell types. A computational model reveals that the band length and the density of sodium channels in the band have the strongest influence on the response to electric stimulation. Most other neuronal properties, e.g., soma size, dendritic field extent, etc. have only a modest effect on threshold. Temporally, most types respond to stimulation frequencies up to ~350 Hz, however some respond to stimulation at frequencies > 500 Hz.

**15. SUBJECT TERMS**
retinal prosthetic, retinal ganglion cell, electric stimulation, initial segment

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 30 | 19b. TELEPHONE NUMBER *(include area code)* |

## Table of Contents

# Introduction

The overall goal of this research project is to develop more effective methods of neural stimulation in order to improve the clinical outcomes associated with retinal prosthetics [5-9]. To accomplish this, we are investigating the mechanism(s) by which different types of retinal neurons respond to electric stimulation. During Year 1 of this grant, we gained significant insight as to the site in ganglion cells that responded to electric stimulation. We found, for example, that positioning the stimulating electrode over the proximal axon, approximately 40 μm from the soma resulted in the lowest threshold for activation. This physiologically identified site was spatially aligned with an immunochemically identified dense band of voltage-gated sodium channels also located in the proximal axon.

Surprisingly, the band properties, e.g. length and location, were different in the few types of ganglion cells we had explored. Therefore, since the band is the site that is responsive to electric stimulation and since the band is different in different types of ganglion cells, it seemed likely that different types of ganglion cells will have different responses to electric stimulation. We confirmed that this was in fact the case: thresholds between different types of ganglion cells were different.

In Year 2, threshold maps have been completed for many additional types of ganglion cells and the sodium channel bands have been analyzed in each of these types as well. This work was described in a publication released during the past year [10]. In addition, our focus has shifted from identifying the site of lowest threshold to trying to understand how the different properties of the band influence threshold. For example, do longer bands underlie lower thresholds? To explore this, we developed a multi-compartment computational model that allows us to explore how individual anatomical properties influence the response to electric stimulation. In this manner we found that the length of the band and the density of sodium channels within the band both had a strong influence on threshold. Band location had only a minor affect. Non-band properties such as soma size and the dendritic field extent were also evaluated and also had little effect. However, the diameter of the axon hillock (the portion of the axon between the soma and the band) had a relatively strong influence on threshold - comparable to that of the band length.

We have also begun to study the temporal responsiveness of ganglion cells. We found that most types respond to very high frequency trains of short duration pulses (200 μsec) – all types generate one spike per pulse at frequencies of 200 Hz and some respond similarly at 350 Hz. Surprisingly, a few types generate one spike per pulse at frequencies as high as 500 Hz. We did not expect this to be the case since spike duration and the absolute refractory period were thought to be longer than 2 ms. Preliminary findings suggest that the sodium channel band is capable of spiking at a higher frequency than that of the soma. We will complete this analysis during the upcoming year and attempt to determine which properties of the band influence the temporal responsiveness of the cell.

# Body

Task 1

Determine activation threshold as a function of electrode position in each type of retinal ganglion cell.
(Months 1-18)

Goals as listed in the approved statement of work:

1. Measure spatial profiles (threshold as a function of distance between stimulating electrode and soma) in each ganglion cell sub-type (Mos. 4-18).
2. Measure threshold for electric stimulation at the soma in each ganglion cell sub-type (Mos. 1-12).

Goal 1

The objective of the first goal was to determine whether 'hotspots' of low threshold exist in retinal ganglion cells, e.g. what position of the stimulating electrode results in the lowest activation threshold. Previous computational [11, 12] and physiological studies [13, 14] each found different sites – these included the soma, axon hillock, initial segment, axon bend and the thin section. Although all identified sites were in or around the soma/proximal axon region, the actual site of lowest threshold remained unknown. Therefore, we mapped threshold as a function of the position of the stimulating electrode allowing us to correlate threshold measurements to specific anatomical regions of the targeted cell.

These 'threshold maps' for a single type of ganglion cell were described partially in last year's update. We have since measured many additional maps in DS cells as well as in six additional types of ganglion cells. A sample map from a BT ganglion cell is shown in Figure 1.

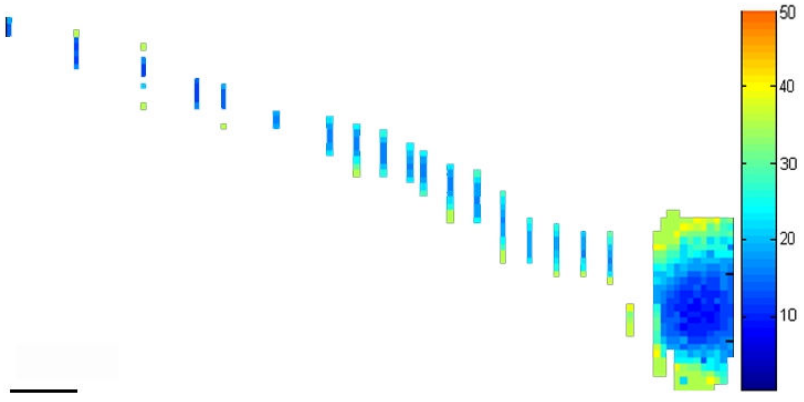


**Figure 1: A sample threshold map:** Threshold map for a BT ganglion cell. Each colored square represents a threshold measurement for that location of the stimulating electrode. Soma is centered at the right edge (bottom pixel). The color bar at right correlates pixel color to the pulse amplitude in μA (threshold). Scale bar: 100 μm.

The overall appearance of the map is highly consistent across all measured types: a single region of low threshold is always offset from the soma (bottom right edge of the map) and centered over the proximal axon. The types of cells in which maps have been obtained include both the largest and smallest dendritic fields as well as from the cells with the highest and lowest spike rates. The consistency of maps across a wide range of different ganglion cell types suggests that maps are qualitatively consistent for all types, and therefore, mapping the remaining types will yield little or no new insights. It is therefore difficult to justify the use of additional rabbits to simply meet the stated goal of 'we will measure spatial patterns of threshold *in each type.'* Instead, we believe that qualitatively similar results in all 6 types tested to date meet the goals of this aim and therefore we consider this Task to be complete.

In addition to the original goals of this task, we identified two different types of G11 (ON and OFF brisk transient). The two types have differences in both their physiological responses (Fig. 2) and their anatomy (see Task 3), and therefore, we completed maps for each type. There has not been a previous report of multiple types of BT ganglion cells and we are working towards a publication that details the differences between the two types – separate from the aims of this grant.

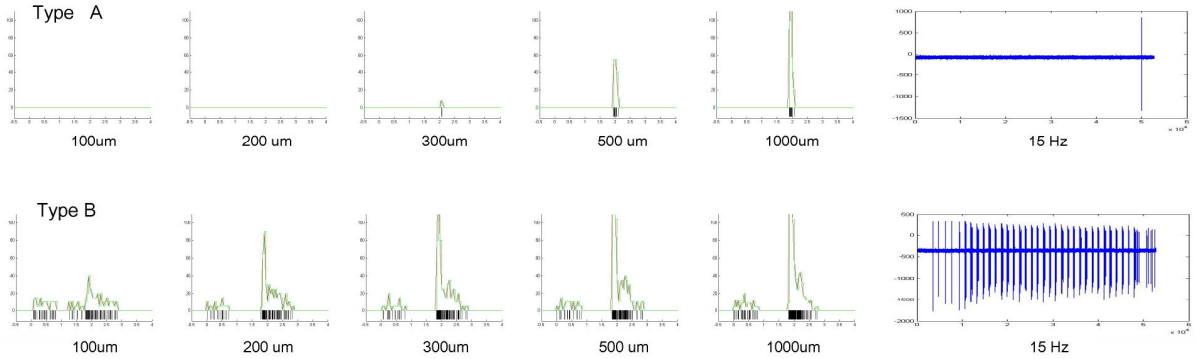


**Figure 2: Physiological responses suggest two sub-types of BT (G11) ganglion cells in rabbit.** Each of the panels represents the response to a light stimulus. Panels labeled by 100 µm – 1000 µm are histograms of the spiking response to a one second flash of a bright square – the size of the square is different for each panel and indicated by the label below each panel. Note that Type B has a robust response to smaller size squares while Type A has little or no response other than for the largest squares. The right most panels are the raw spiking trace in response to a 1000 µm square flashed at 15 Hz (square wave profile). Type B cells generated spikes at the offset of each phase of the stimulus. Type A had almost no response – typically a few spikes or a brief burst at the end of the stimulus.

<u>Goal 2</u>

The objective of this goal is to explore the differences in how each type of ganglion cell responds to electric stimulation. We are particularly interested in whether different types of ganglion cells have different thresholds. Ongoing work suggests that this is the case and so we want to understand the nature of these differences. For example, the type with the lowest threshold in the human retina is most likely to have the strongest influence on clinical responses. If this is not one of the types

thought to mediate conscious vision (e.g. midget or parasol), it could help to explain the lack of consistent, high-quality percepts during clinical trials. We also want to determine why and how threshold differences arise as a basis for developing new methods that can selectively activate individual types.

Originally, we planned to measure somatic thresholds (stimulating electrode positioned directly over the soma) as the basis for comparing thresholds between types. However, the threshold maps from all types revealed that the lowest thresholds occurred when the stimulating electrode was offset from the soma. Further, the relative offset of the low threshold region was somewhat random (it was always oriented to the optic disk side of the retina, but its exact location ranged almost the full 180° within that side). Because the electric field arising from our stimulating electrode was not perfectly symmetric, threshold measurements when the stimulating electrode was positioned at the soma over-estimated threshold if the low threshold region was in one direction and under-estimated threshold if the low threshold region was in the other direction. Therefore, we did not feel that somatic threshold was the appropriate parameter with which to perform comparisons and instead, we compared the lowest thresholds across cells (threshold measured at the center of the low threshold region). A comparison of thresholds for three different cell types is shown in Figure 3. More details on this finding are also included in our recent publication [10].

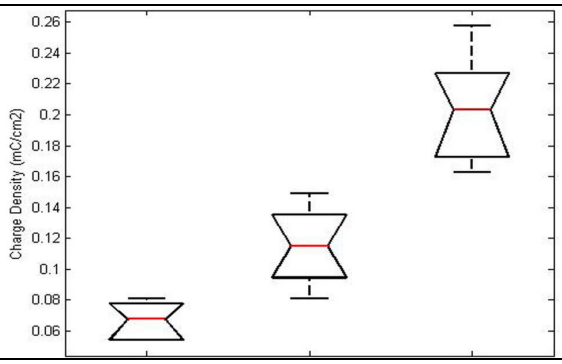


**Figure 3: Threshold differences for cell types**. Population results from threshold measurements in BT (left, n=7), DS (middle, n=10) and LED (right, n=5) ganglion cells. The red horizontal lines indicated the median threshold for each type; the upper and lower horizontal edges of the box represent the upper and lower quartiles. The whiskers indicate the lower and upper extents of the data for each type. Thresholds were statistically different for all three types.

We are most interested in the underlying cause of the threshold differences. As mentioned in last year's report, as well as in Tasks 3 and 4 (below), the center of the low threshold region is spatially aligned with an immunochemically identified dense band of sodium channels. There are differences in the properties of the sodium channel bands for each type suggesting that the band properties underlie the threshold differences. We are exploring this possibility in detail via two approaches: (1) use of a multi-compartment model and (2) correlative analysis between our physiological and anatomical measurements (see Task 4). A thorough understanding of the response differences is likely to underlie the development of powerful new stimulation methods that can create patterns of neural activity that more closely resemble normal physiological patterns.

<u>Year 2 Summary for Task 1</u>

- Goal #1: We have completed maps for different types of ganglion cells.

- In all cases, maps were qualitatively similar: a single region of low threshold was found in the proximal axon region. The center and size of the low threshold region varies across types. As we describe for Tasks 3 and 4, the properties of the low threshold region are correlated to a dense band of voltage gated sodium channels that were identified using immunohistochemistry.
- We now consider goal #1 of this task to be complete.
- Goal #2: As discussed in the body of this task, we no longer believe that somatic threshold is the ideal parameter with which to compare thresholds between types. Instead, measurement of lowest threshold (center of the low threshold region) will provide the most meaningful comparison. This has been completed in seven different types of ganglion cells.
- We are still processing the threshold data from four of the measured types. This data will be analyzed by September 2009 at which point we will consider goal #2 of this task to be complete.
- We are actively studying why the threshold differences arise. It seems likely that specific properties of the sodium channel band play a role and we are exploring this possibility in Task 4.

## Task 2

Determine the temporal response properties for each ganglion cell sub-type.
(Months 10-21, erroneously listed as 4-14 on approved SOW)

Goals as listed in the approved statement of work:

1. Determine the maximum rate for which one spike per pulse is elicited in each ganglion cell sub-type. Months 10-21
2. Determine the response to high frequency stimulation (>250 Hz.) in each ganglion cell sub-type. Months 10-21

An earlier study of ours [15] revealed that short duration stimulus pulses reliably elicited one spike per pulse. Following this, it was found that high-frequency trains of short pulses continued to elicit one spike for each pulse and therefore, could be used to create specific patterns (frequencies) of spiking. However, it is not clear whether this method can in fact be used for all types of ganglion cells and so goal 1 proposes to evaluate the one spike per pulse paradigm for all types of ganglion cells.

The maximum spike rate for retinal ganglion cells is thought to be ~300 Hz – this corresponds to a spike duration of approximately 2 ms and an estimated absolute latency period of 1 ms. Therefore, the question arises as to how ganglion cells will respond to stimulation frequencies > 300 Hz. This will be tested as part of goal 2.

As mentioned in last year's report, we have put considerable effort into Tasks 1, 3 and 4 and as a result, our efforts with this task have been delayed. We are now performing these experiments and will complete this task during the final year of the grant.

Goal 1

Sample plots that illustrate our findings are shown in Figure 4. Stimulation consists of 200 μsec biphasic pulses with uniform spacing between all phases. For example, if the stimulation frequency is 250 Hz, the total period is 4 ms and successive cathodal and anodal phases are separated by 2 ms. Measurements are made in voltage clamp mode and therefore we measure currents (not voltages) which have inverted polarity (cathodal pulses are upward, anodal pulses are downward). The easiest way to observe spikes in these traces is to look at the transition from one upward deflection (a single cathodal pulse) to the subsequent downward deflection (a single anodal pulse). When this is compared to the transition from the downward deflection to the upward deflection, an asymmetry is observed; the complex waveform that follows the cathodal pulse is the action potential. The large upward deflection (arrows) that occurs consistently after each cathodal pulse is the after hyperpolarization (upward because of voltage clamp). At 344 Hz, one spike is elicited for each pulse (Fig. 4a). At 384 Hz in the same cell, each pulse no longer elicits a spike (Fig. 4b).

In another type of ganglion cell (G10, ON DS cells), stimulation frequencies up to 526 Hz pulses elicit one spike per phase (Figs. 4c, d, respectively). Interestingly, the amplitude of the elicited spike is seen to decrease during the first few stimulus pulses at 526 Hz (Fig. 4d). This is most likely the result of inefficient back propagation: the action potential is initiated in the sodium channel band and propagates retrogradely to initiate a spike in the soma. The somatic action potential is large but if the band action potential does not give rise to a somatic action potential, the size of the action potential measured at the soma is small. Thus, it is likely that the band is capable of responding at a much faster rate than the soma. These results also suggest that some types of ganglion cells, e.g. G10, are capable of responding to a higher stimulation frequency than other types. Different types are known to have different sodium channel bands suggesting that specific properties of the band may underlie the response properties of the cell. This will be explored further as part of Task 4.

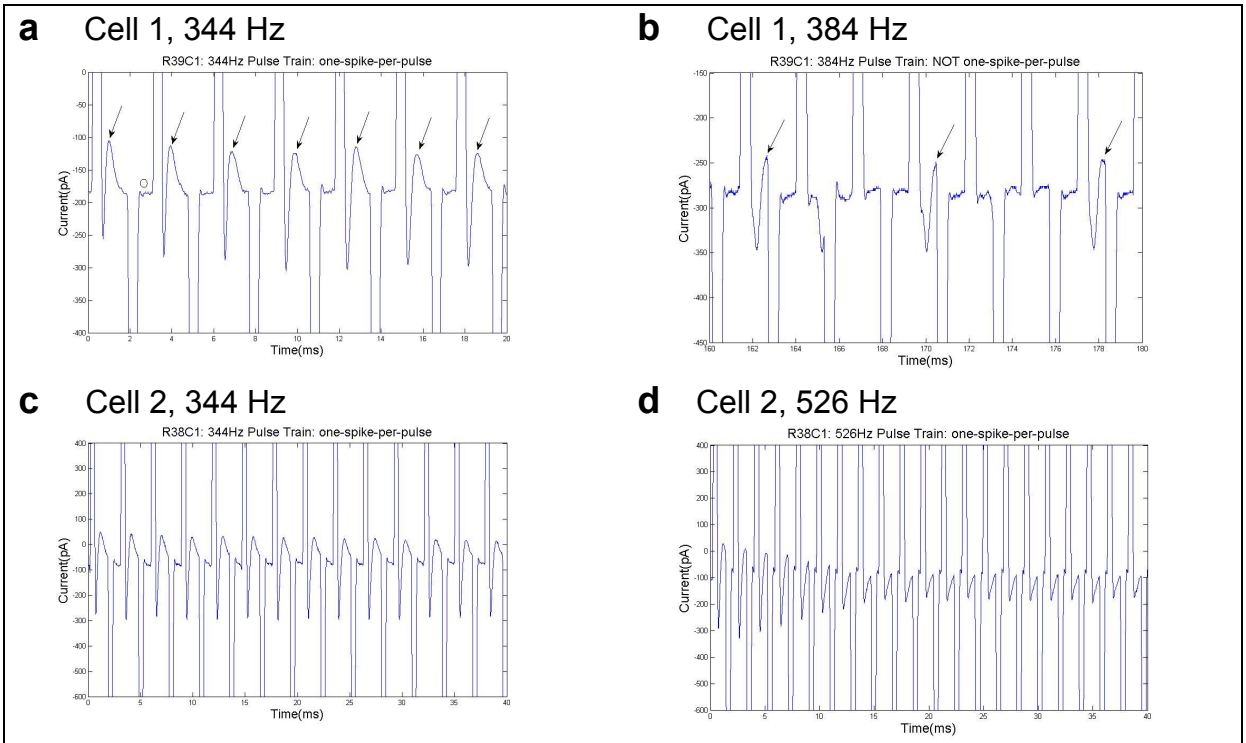


**Figure 4: Measurements of high frequency stimulation with short pulses.** Each trace is the voltage clamp response to a series of 200 µsec rectangular pulses at different frequencies. Comparison of the waveform at the transition between anodic and cathodal (downward deflection to upward) vs. the transition between cathodal and anodic (upward deflection to downward) reveals the action potentials that follow the cathodal phase. (**a**) Responses to 344 Hz stimulation. Cathodal pulses (upward deflections) elicit action potentials – the arrows point to the peak of the after hyperpolarization (the depolarizing phase is obscured somewhat by the artifact associated with the trailing edge of the cathodal pulse). Anodic pulses (downward deflections) do not elicit action potentials (circle indicates the first anodic pulse). (**b**) Response to 384 Hz stimulation in the same cell as (a) reveals that only some of the pulses elicit action potentials (arrows). (**c**) Response to 344 Hz stimulation in a second cell again reveals one spike elicited for each cathodal pulse. (**d**) Response to 526 Hz also reveals one spike per pulse, although pulse amplitude is seen to decrease during the first few pulses and then remain constant.

<u>Goal 2</u>

Originally, we did not expect ganglion cells to respond to electric stimulation at frequencies much greater than 250 Hz. The duration of a single action potential is typically > 2 ms plus the addition of an absolute refractory period suggested that spikes might not be generated any faster than 250 or 300 Hz. However, we were interested in whether other presynaptic neurons or possibly even other spiking mechanisms might be activated by stimulation at frequencies higher than the maximum spike rate.

We are still exploring this. Our unexpected finding that some neurons respond to direct stimulation at rates >500 Hz (Fig. 4d) has caused us to test even higher frequencies of stimulation as part of goal 1 than we originally anticipated. A sample test from 1000 Hz sinusoidal stimulation is shown in Figure 5. Stimulation was delivered via an electrode positioned 25 µm above the soma. Surprisingly, we found that the ganglion cell responded somewhat periodically to this type of stimulation, generating one action potential approximately every 7 periods. There are several mechanisms by which this may occur and we are currently exploring these.

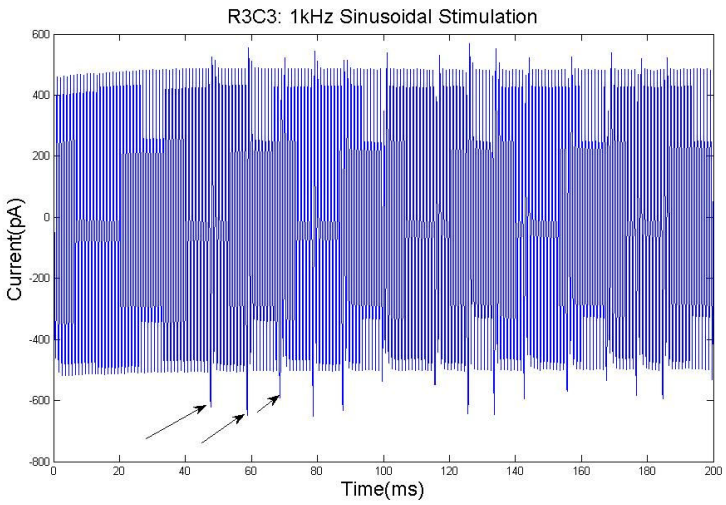


**Figure 5: High frequency stimulation activates intermittent spiking.** The response to stimulation at 1000 Hz. Spikes (arrows) were elicited although they were intermittent.

Summary: Task 2

- As stated previously, delays in this task arose from additional effort we have place in completing Tasks 3 and 4 and in publishing the work we've done to date.
- We have completed many of the measurements associated with Goal 1 and anticipate completion by October 2009.
- Goal 2 has begun and we are testing frequencies up to 1000 Hz. We anticipate completion of this goal during the final year of this grant.

## Task 3

Measure the distribution of VGNaCs in each ganglion cell sub-type.
(Months 1 -27, erroneously listed as 9-18 in approved SOW)

Goals as listed in the approved statement of work:

1. Measure the distribution of voltage-gated sodium channels in each ganglion cell sub-type using immunochemistry. Months 1-24.
2. Determine which sub-types generate action potentials in their dendrites (evaluates functional viability of localized voltage-gated sodium channels). Months 19-27.

The overall objective of this task was to measure the distribution of voltage-gated sodium channels in each ganglion cell type. One of our original hypotheses was that regions of low threshold obtained physiologically would be correlated with anatomical sections of the cell that contained high levels of sodium channels (obtained immunochemically). Many other possible sources for the low thresholds (e.g. the soma and axon bend) were quickly eliminated (see Task 4), increasing the likelihood that our original hypothesis was valid.

Goal 1

We used immunochemical methods to determine the distribution of voltage gated sodium channels in ganglion cells. A complete description of our methodology can be found in the Methods section of our recent publication [10]. Briefly, we are localizing sodium channels using either Pan Sodium Antibody (PAN), an antibody that recognizes all isoforms of neuronal sodium channels [16], or by using antibodies for Ankyrin G, a structural protein associated with high density regions of voltage-gated sodium channels and typically found in the initial segment and nodes of Ranvier [17-19]. Our techniques are similar to those of previous groups that have explored sodium channels in retinal neurons [19-21]; these efforts have been greatly assisted by our collaborator Richard Masland, along with Tatjana Jakobs, Amane Koizumi and Bin Lin, post-doctoral fellows in his laboratory.

Sodium channel distribution in DS ganglion cells

The immunochemically identified sodium channel band is shown in Figure 6 (left) (repeated from last year's report). We characterized the physical properties of the sodium channel band by measuring the length (distance between the vertical lines) as well as the distance between the soma and the closest edge of the band. We plotted band length vs. band distance for 17 different DS ganglion cells (Fig. 6, right). For DS cells, the average band length was 28.6 ± 4.8 μm and the average distance from the soma was 22.8 ± 6.4. Generally, the band properties appear to 'cluster' together suggesting that the band length and location are consistent within this type and therefore, further suggesting that these specific properties may serve a functional role.

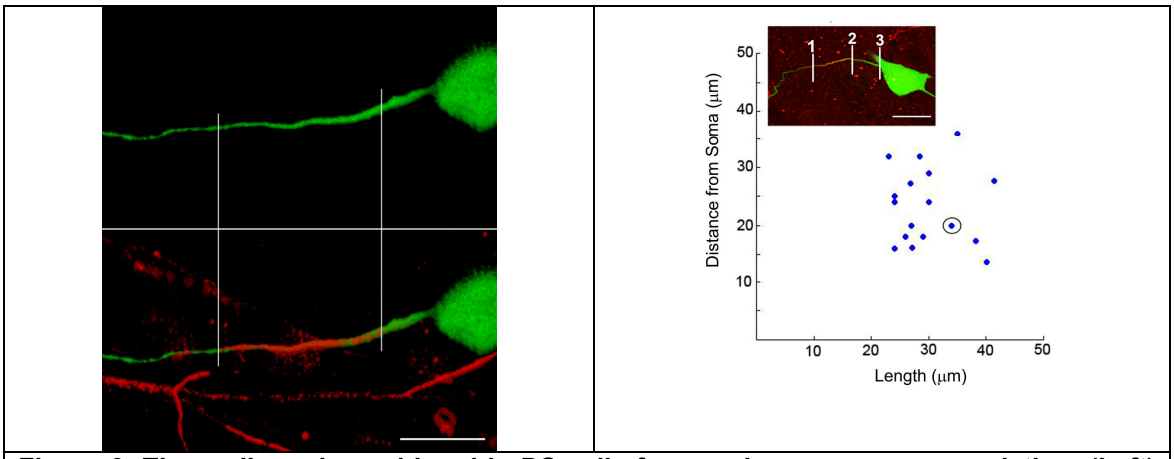


**Figure 6: The sodium channel band in DS cells forms a homogeneous population. (Left)** The dense sodium-channel region is co-localized to the region where the axon diameter decreases. *Top*: The soma and axon of a DS cell (green) that has been filled with a fluorescent dye. *Bottom*: The same DS cell (green) after immunostaining for PAN (red). One of the long immunostained bands (red) is coextensive with the DS axon. The other bands (red) are presumably associated with other (unfilled) ganglion cells. The vertical lines indicate the approximate extent of the dense sodium-channel region. Extension of the vertical lines up to the top panel reveal that the dense sodium-channel region is in a portion of the axon where the diameter transitions from large to small (compare the axon diameter at the right and left lines). Scale bar: 20 μm. **(Right):** Length and location analysis of high-density sodium-channel regions in DS cells suggests a homogeneous population. The length of the region (inset, distance between 1 and 2) was plotted vs. the distance between the soma and the proximal edge of the region (inset, distance between 2 and 3). Each point is from a different cell. The circled point is from the cell shown in the inset. Inset scale bar: 25 μm.

Sodium channel distribution in other ganglion cell types

Similar to the analysis of DS cells, we plotted band length vs. band distance for other types of ganglion cells (Fig. 7a). Similar to DS cells, the band properties from other types appear to cluster together as well. This finding had not been reported previously and has very important implications for both normal retinal function as well as for retinal prosthetics. These implications are discussed in subsequent sections. To further explore the differences in sodium-channel bands between types, we measured the bands in 19 additional unidentified ganglion cells (Fig. 7, right). Band properties from unidentified cell types overlapped the band properties from identified types suggesting that band properties are not unique for all cell types. Across the population, band lengths and locations were bounded: lengths ranged from 10 to 50 μm, whereas distance from soma ranged from 0 to 40 μm.

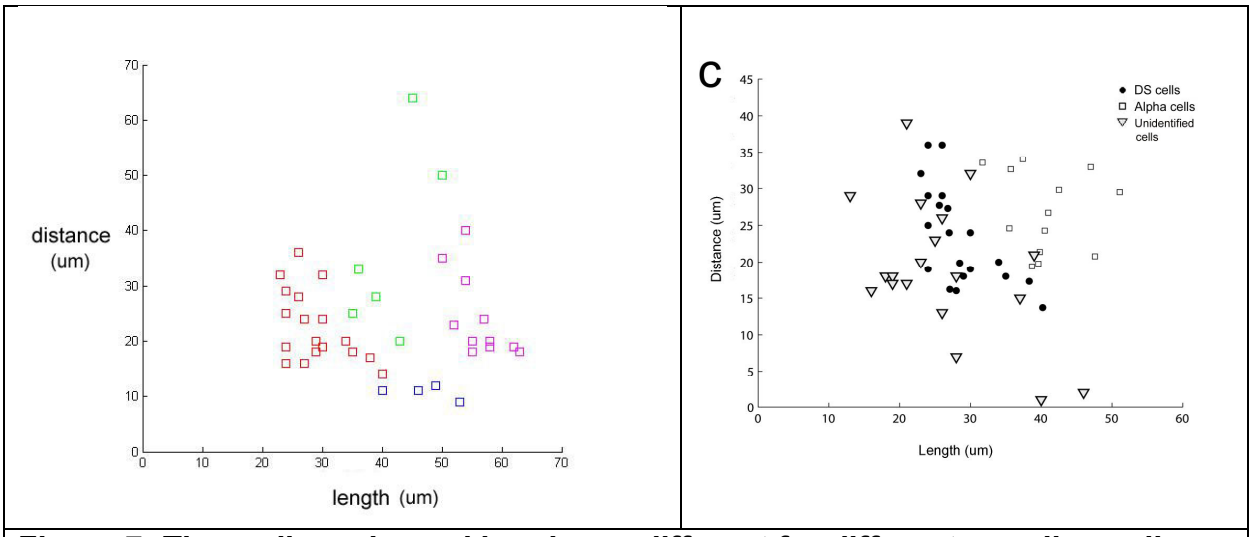


**Figure 7: The sodium channel bands are different for different ganglion cell types. (left)** Similar to the data from Figure 6, each point represents the length and location of a measured sodium channel band. Red squares: ON-OFF DS cells; Blue: LEDs; Green: ON DS cells; Pink: BT cells. (**right**) Measurements of sodium channel bands from 19 unidentified types of ganglion cells overlaid on DS and alpha cells.

Role of sodium channel band differences in normal function (light responses)

Under normal conditions, the action potential is thought to be formed in the proximal portion of the ganglion cell axon (the initial segment), most likely within the dense band of sodium channels. It is tempting to speculate therefore that the differences in band properties contribute somehow to the different spiking responses observed in the different types of ganglion cell [22, 23]. Variations in band properties could modulate a wide range of properties related to the spiking response, e.g. latency and/or spiking frequency. Although this is outside the original aims of this grant, we have begun to explore this via the use of a multi-compartment model created in NEURON [24]. Briefly, we found that band properties do in fact modulate the spiking response of ganglion cells (Fig. 8). We compared a short band that was far from the soma to a long band that was close to the soma; all other parameters in the 2 models were identical. We found that both the amount of synaptic input required to elicit spikes (threshold), as well as the spike frequency in response to a given stimulus were different (Fig 8, top and bottom, respectively). While many other morphological and physiological properties are likely to contribute to the differences in spike patterns exhibited by each type, our results strongly suggest that band differences contribute as well. We continue to explore outside the original aims of this grant.

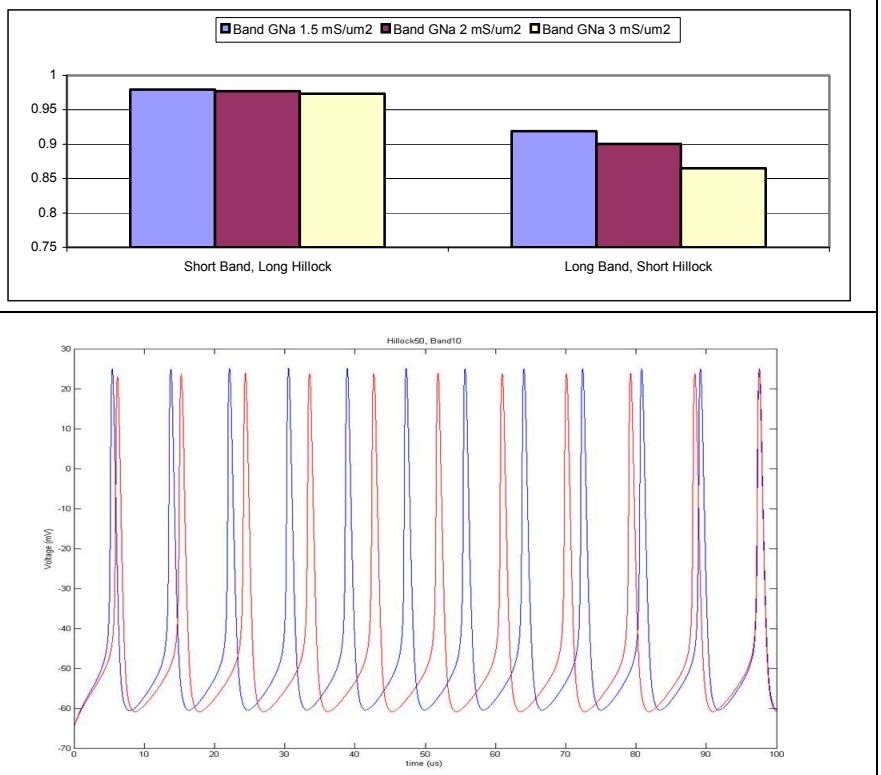


**Figure 8: Band differences modulate the spiking response of ganglion cells.** (Top) The threshold required to elicit an action potential in a model cell with a short band that was far from the soma (left) vs. a cell that was identical except the band was long and close to the soma (right). Individual bars represent three separate runs for each configuration (different sodium channel conductances). (Bottom). Response of the same 2 models to a suprathreshold stimulus. The long band that was close (blue) generated action potentials at a higher rate than the cell with the short band that was far (red).

Role of sodium channel band differences in shaping the response to stimulation

Since the band is likely to be the site that is responsive to electric stimulation, band differences will probably influence the response to extracellular stimulation. This has been explored in some detail, also via use of a multi-compartment model and is described below in Task 4.

Goal 2

The objective of Goal 2 is to use physiological methods as a higher sensitivity detector of dendritic sodium channels. Several physiological studies indicate that retinal ganglion cells generate action potentials in their dendrites [25, 26], strongly suggesting the presence of voltage-gated sodium channels in the dendrites. The presence of sodium channels in the dendrites raises the possibility that they can be activated with electric stimulation [12] and therefore, we wanted to determine whether these channels can be used to effectively activate ganglion cells. Following the methods from one of these studies [25], we are able to similarly detect dendritic action potentials in DS ganglion cells (data not shown) even though we could not immunochemically detect the channels.

Initial testing revealed that the possibility of activating dendritic action potentials was not likely. Dendritic action potentials were observed via use of a pico-spritzer that applied tetrodotoxin (TTX), a blocker of voltage gated sodium channels, to a small focal region around the soma. This blocked somatic action potentials and allowed us to more clearly visualize synaptic currents. We found dendritic action potentials in two types of ganglion cells. In agreement with previous results from Oesch et al [25],

directionally selective ganglion cells exhibit dendritic action potentials (data not shown). This is despite the fact that the threshold map data gives no indication that any portion of the dendritic field is sensitive to electric stimulation (see the maps in Figures 2 and 6 of reference [10], or in last year's summary). We also found dendritic action potentials in BT ganglion cells but again there was absolutely no indication of sensitivity to electric stimulation in their dendritic field (see Fig. 1). These findings suggest that dendritic sodium channels are not very sensitive to electric stimulation. This is not very surprising however based on our immunochemical studies: the density of sodium channels was high in the sodium channel band and very low in the dendrites. Since the presence of sodium channels in the dendrites does not have any sizable influence on the response to electric stimulation, we do not plan to pursue additional follow-up efforts related to this goal.

Summary: Task 3

1. We have completed the voltage-gated sodium channel distribution in retinal ganglion cells (Goal #1).
2. We have identified two ganglion cell types that exhibit dendritic action potentials and therefore, presumably contain voltage-gated sodium channels in their dendrites (Goal #2). Identification of these channels did not lead to a new method of stimulation - probably because of sparse dendritic distribution.

## Task 4

Correlate dendritic field extent, soma size and distribution of VGNaCs with the activation threshold and dynamic range measurements described above. (Months 19-36)

Goals as listed in the approved statement of work:

1. Correlate somatic threshold with dendritic field extent and distribution of voltage-gated sodium channels with the activation threshold and dynamic range measurements described above. Months 19-36.
2. Correlate temporal response properties with dendritic field extent and soma size in each ganglion cell sub-type. Months 21-27.
3. Correlate spatial profiles with voltage-gated sodium channel distribution in each ganglion cell sub-type. Months 28-33.
4. Correlate spatial and temporal response properties with other morphologic features in each ganglion cell sub-type. Months 34-36.

The ultimate goal of this study is to learn how electric stimulation initiates spiking responses in retinal ganglion cells so that we can develop new methods that provide better control of elicited neural activity. Each of the goals in this task explores the relationship between one or more properties of the ganglion cell and its response to electric stimulation.

Goal 1

One of the most fundamental questions we seek to answer is which properties of the ganglion cell underlie the cell's sensitivity to electric stimulation. Our preliminary data (submitted with the grant application) suggested that threshold differences were correlated with the size of the dendritic field. However, we recognized early on that threshold was also correlated with soma size and axon size, raising the question of which property had the strongest influence.

Our studies to date have significantly clarified our understanding of which neuronal properties influence the response to electric stimulation. We now believe that the dense band of sodium channels in the proximal axon is the neuronal element that responds to electric stimulation and variations in band properties are likely to underlie the differences to electric stimulation. However, it is likely that other elements of the cell influence threshold as well. For example, it is well known that the diameter of an axon influences its sensitivity to electric stimulation with larger diameter axons having the lowest thresholds. Therefore, it is likely that in addition to band properties such as length and distance from the soma, the diameter of the axon at the location of the band may also modulate threshold.

Unfortunately, it is not easy to test the sensitivity of individual properties since individual properties do not vary independently across ganglion cell types. Therefore, to get a preliminary sense of which neuronal elements had the strongest influence on

sensitivity, we built a multi-compartment model (Fig. 9) that allowed us to test the effect of individual neuronal elements. The model was developed similar to previous studies [11, 12, 27] but included the anatomical and biophysical properties identified by our studies [10]. For example, each neuron included a sodium channel band in the proximal axon and the axon diameter tapered in this same region as well.

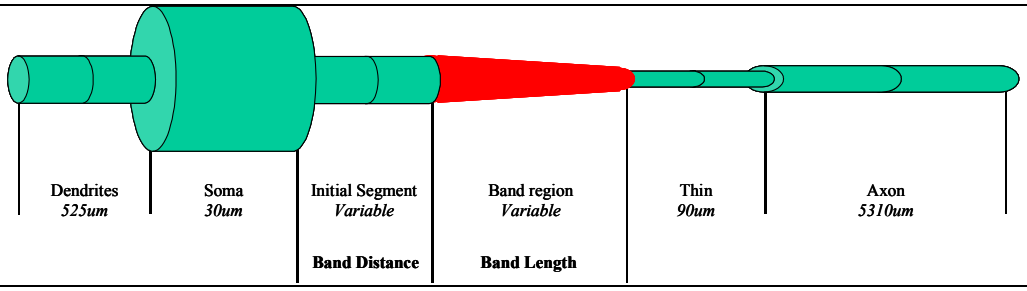


**Figure 9: Development of the simplified multi-compartment model.** A model ganglion cell was constructed from multiple elements. Each compartment was 6 μm long; multiple compartments were assembled as needed to reach the total length of each element. The diameter and biophysical properties (e.g. sodium channel density, $G_{Na}$) vary independently in each compartment. To study the effect of increasing the length of a particular region, i.e. soma, more segments were added to the soma region. To study the effect of varying the sodium channel density in a specific portion of the cell, $G_{Na}$ of the appropriate segments was varied.

To explore the role of a single neuronal element, we fixed all parameters of the model cell except for the one under evaluation. A sample result for the evaluation of band length is shown in Figure 10. We measured threshold of the model cell for an electrode positioned at many different locations along the cell. Consistent with our physiological measurements, we found that threshold was lowest when the stimulating electrode was positioned above the sodium channel band. In particular, threshold was lowest when the stimulating electrode was directly above the most distal compartment of the band – suggesting that the distal edge of the band is the site of spike initiation in response to electric stimulation (and possibly in response to synaptic input as well). When the length of the band was increased, the site of lowest threshold remained at the distal edge of the band however, absolute threshold decreased for longer bands (compare the threshold levels indicated by the dotted horizontal lines for the shortest and longest bands). This suggests that longer bands are associated with lower thresholds.

We explored the sensitivity of band location in a similar manner (Fig. 11) and found that if the distance between the sodium channel band and the soma was very small, the threshold for eliciting action potentials was increased (compare the two arrows on the left of Fig. 11). Threshold was reduced as the distance increased. However, once the distance between band and soma increased above ~20 μm, there were no further reductions in threshold (compare the two arrowheads). We were able to test the effects of other band and non-band properties in a similar manner allowing us to compare the relative sensitivity of individual parameters.

As expected, we found that certain band properties had strong influences on threshold while others do not (Fig. 12). For example, band length and the density of

sodium channels had the strongest influences while band location had a weaker effect, especially for more distant locations. Amongst non-band properties, band diameter had the strongest influence while the size of the soma, the size of the dendritic field and properties of the distal axon had milder effects.

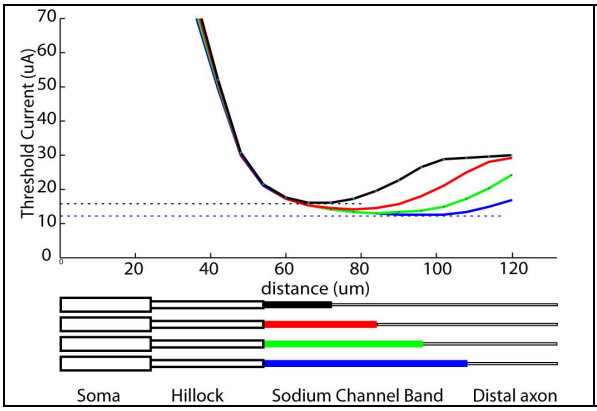


**Figure 10: Longer bands are associated with lower thresholds.** Threshold was determined as a function of the position of the stimulating electrode for four different lengths of the sodium channel band. The lowest threshold for each of the four trials was different; the longest bands resulted in the lowest thresholds. Note that the lowest threshold occurs when the stimulating electrode is positioned over the distal portion of the band, regardless of band length.

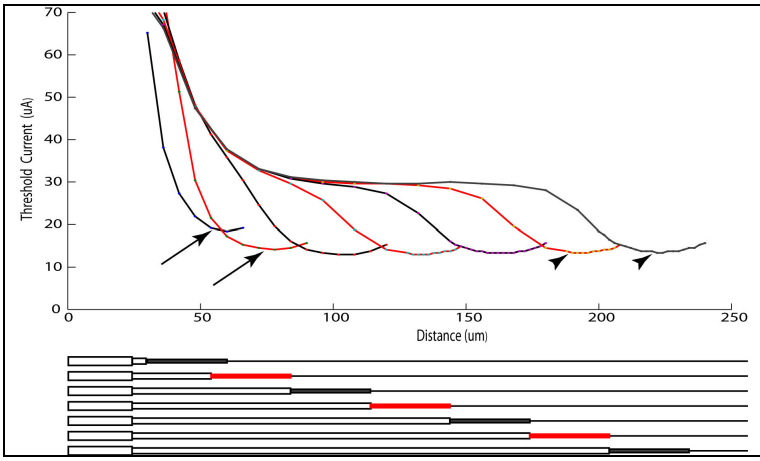


**Figure 11: Band location affects threshold only when close to the soma.** Threshold was determined as a function of the position of the stimulating electrode for seven different simulations. In each successive simulation, the length of the axon hillock was increased in order to move the sodium channel band further from the soma. The schematic elements (bottom) are identical to those of Figure 10.

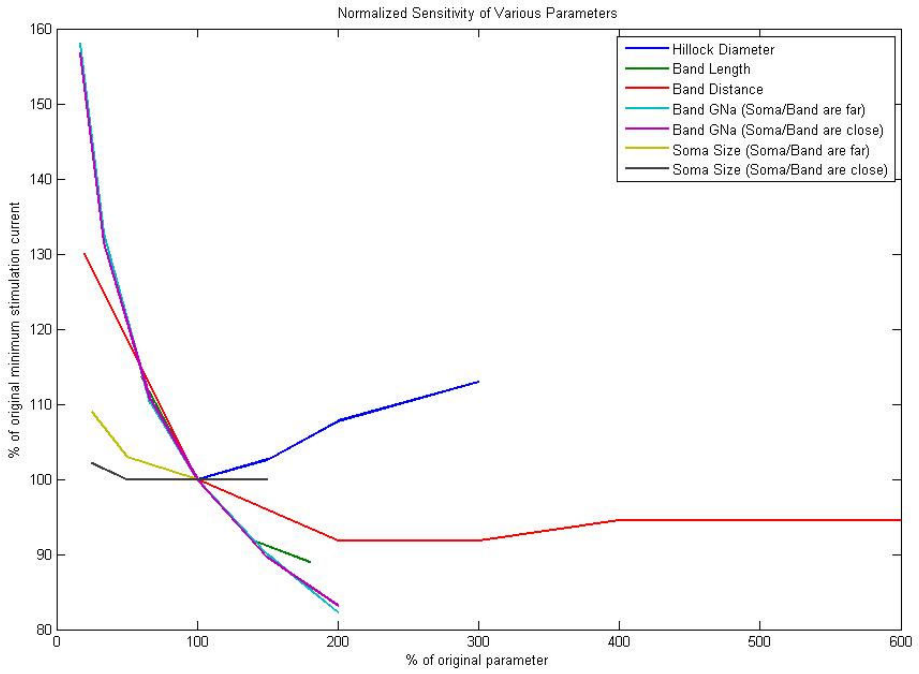


**Figure 12: relative sensitivity of band properties.** Each line represents the sensitivity of one parameter to changes in that parameter. For example, a line with a negative slope suggests that threshold decreases as the parameter increases. The slope of the trace indicates the approximate sensitivity of that parameter. Identification of each parameter is given in the legend. The x-axis represents the normalized change of the parameter where the base model is located at 100%. The y-axis represents the percent change in stimulation current.

During the upcoming year, we will expand our study of which neuronal elements influence threshold by correlating physiological measurement of threshold to measured anatomical properties. By collecting both measurements in a large number of cells we hop to be able to confirm the correlations revealed by the computational model. To do so, we will restrict physiology experiments to a simple determination of minimum threshold (at the band) and then attempt to break in to the cell via whole cell patch clamp in order to perform immunochemistry and capture the band properties.

Understanding which features underlie threshold will help us to better understand and control clinical stimulation. For example, knowledge of the specific features that influence threshold will allow us to assess why ganglion cell thresholds increase in degenerate animal models. We will be able to determine which specific features are changing. In separate studies we are exploring how to optimally stimulate ganglion cells based on the properties of the band. This knowledge, coupled with an understanding of how these properties change during degeneration will allow us to optimize stimulation for ganglion cells in a degenerate retina.

<u>Goal 2</u>

Our preliminary data suggests that different types of ganglion cells have different maximum response frequencies, e.g. continue to respond with one spike per pulse to different levels of stimulation frequency. It is likely that the properties of the sodium channel band influence these differences (in addition to the threshold differences described earlier). We will perform further analysis after we have completed the temporal response measurements described in Goal 2. We expect this to be completed towards the end of the upcoming year.

Goal 3

Much of the work for this goal was presented in preliminary form in last year's update. Figure 13 shows the threshold map and the alignment of the physiological responses with the targeted cell that was included in our publication [10]. The close alignment (Fig. 13c) indicates that the sodium channel band is the site of lowest threshold and most likely the site of spike initiation as well. We now have similar findings in many additional ganglion cells (and also in many different types) and the results are consistent. As mentioned earlier, we have had some difficulty in un-equivocally identifying each and every type of ganglion cell. However, we no longer feel that this is necessary since the data is very consistent across the wide range of types we have measured to date. We therefore feel that we have clearly identified the site of lowest threshold in retinal ganglion cells – and probably in many other types of CNS neurons as well. An article describing these results was published earlier this year [10]. We consider goal 3 to be complete.

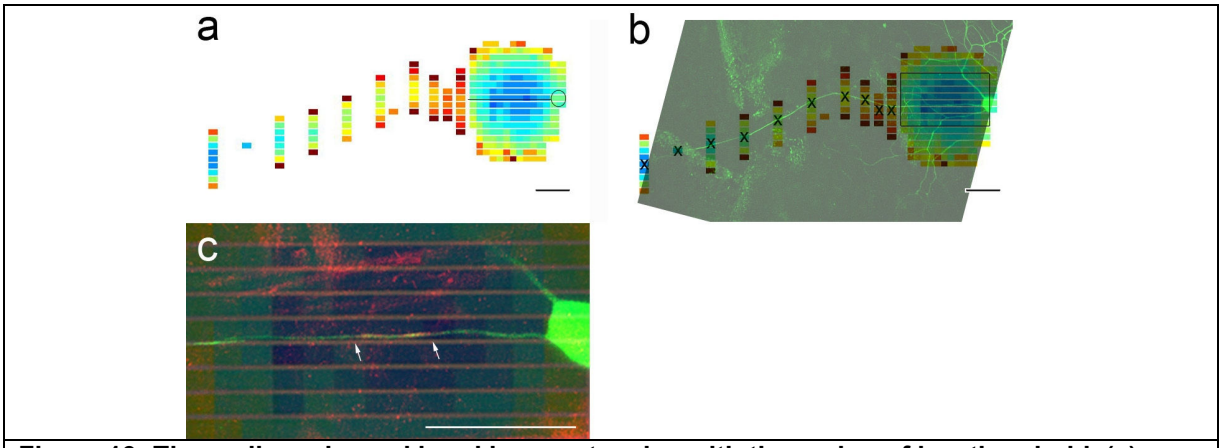


**Figure 13: The sodium channel band is co-extensive with the region of low threshold. (a)** Threshold map of a DS ganglion cell. The map includes threshold measurements over the soma/proximal axon region (right) as well as along more distal sections of the axon (columns in the middle and the left). The threshold values given by each color are identical to those in Figure 2a. The circle indicates the position of the soma. (**b**) Overlay of the threshold map with the dye-filled ganglion cell. 'X's indicate the position of lowest threshold in each column and were aligned to the corresponding portion of the distal axon in the dye-filled cell. **(c)** A higher magnification view of the soma/proximal axon region from (b) (indicated by the rectangular box in (b) ). The position of the sodium channel band, indicated by the arrows, is in the approximate center of the low threshold region. Scale bar in (a), (b) & (c): 50 µm.

Goal 4

This goal is somewhat anti-climatic since we now know that the sodium channel band is correlated with the region of low threshold; other neuronal elements are not well correlated. However, as part of the formal analysis that we did to confirm this, we were able to show that other neuronal elements, especially those previous suggested as the site of lowest threshold, are not in fact the correct site. The data presented here is similar to last year's report. This work was included in our publication [10].

Low thresholds do not arise from the soma or axon bend

The region of low threshold in DS ganglion cells is offset from the soma. This finding is based on the threshold maps from six DS ganglion cells and is summarized in Figure 6 from last year's report (left, blue lines). The average of all low threshold regions is centered ~40 μm from the edge of the soma. In addition, all bands are offset from the soma by at least 20 μm. Taken together, this suggests that the soma is not the source of low thresholds.

To study the alignment between the axon bend and the region of low thresholds, we obtained confocal image stacks of 18 DS ganglion cells. These images allowed us to view the three dimensional morphology, and therefore to determine the location of the axon bend. Figure 14 provides a typical cross sectional view of a DS ganglion cell soma as well as the emerging axon and bend (arrow). In 16/18 DS cells, we found that the bend occurred within 6 μm of the edge of the soma; in the cell of Figure 14, the bend (arrow) actually occurs before the edge of the soma. We found that the center of the low threshold region was typically 40 μm from the edge of the soma in these cells. Therefore, it was typically > 30 μm from the axon bend suggesting that the bend is not spatially correlated with the low threshold region.

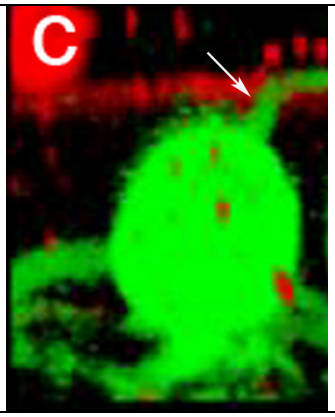


**Figure 14: The axon bend occurs within 6 μm of the soma in DS cells.** A DS ganglion cell soma (green) extends dendritic processes down into the inner plexiform layer and a single axon up to the nerve fiber layer (red). The axon emerges from the vitreal end of the soma (arrow) and ascends directly to the NFL. The bend occurs within the boundaries of the soma. Image width: 40 μm.

Summary: Task 4

- The multi-compartment model has been developed. We are now testing controls by comparing model predictions to existing physiological measurements. Once completed we will publish much of the modeling work described here. We expect this to occur in Fall 2009.
- Correlation with experimental results (band properties vs. anatomy) will take place during the upcoming year.

- Goal 2 is in progress and is expected to be completed during the upcoming year.
- Goal 3 is complete. We have demonstrated the sodium channel band in the proximal axon is the site of lowest threshold. This site is also probably the site of spike initiation and will be tested in future work.
- The site of low threshold has been compared with other neuronal elements; no correlation was found. We will also compare the temporal responsiveness to anatomical regions to see if different areas of the neuron underlie this part of the response. This will be completed during the upcoming year.

# Key Research Accomplishments

Year 1

1. We have shown that threshold levels are lowest when the stimulating electrode is positioned over a portion of the proximal axon.

2. We have shown that the region of low threshold does not correspond to either the soma or the axon bend. Our results directly refute findings from two recent computational studies that each suggested that thresholds were lowest in one of these two regions.

3. We have shown that the region of low threshold corresponds to a dense band of sodium channels located within the proximal axon.

4. We have shown that the sodium channel band corresponds to a portion of the axon in which the diameter tapers from relatively large section associated with the axon hillock to the relatively thin section originally described by Carras et al.

5. We have shown that the sodium channel bands are consistent within a given type of ganglion cell but the bands vary from type to type. There is some overlap in band properties between different types. These difference may play a role in the different spiking responses (to light) generated in different ganglion cells. The differences are also likely to shape the response to electric stimulation and therefore different ganglion cell types may respond differently to electric stimulation.

6. We have shown that the threshold maps are also different across ganglion cell types; these differences presumably arise from the differences in the sodium channel bands.

7. We have shown that thresholds in the distal axon are somewhat variable and can be quite low – even lower than the levels found in the proximal axon. This finding contradicts findings from the two computational studies mentioned earlier (#2), as well as a physiological study, all of which suggest that the axonal thresholds are higher than those of the soma/proximal axon region.

Year 2

1. We have had our first publication from this study (on the alignment between threshold maps and sodium channel bands) accepted and released.

2. We have completed threshold maps in many additional types of retinal ganglion cells.

3. We have identified the sodium channel band differences in many different types of ganglion cells.

4. We have shown, via a computer model, that specific properties of the sodium channel band influence the response to electric stimulation. In particular, we found that band length modulates threshold with longer bands having the lowest thresholds. In addition, we determined that the sodium channel conductance of the band also had a strong influence on threshold.

5. We are developing a manuscript describing these results and anticipate submission in Fall 2009.

6. Our preliminary results indicate that different types of ganglion cells have different maximum spiking levels to short pulses. Nearly all types can respond at rates exceeding 200 Hz and many types can respond at rates exceeding 300 Hz. At least one cell type can respond at > 500 Hz. We are investigating whether specific band properties underlie the difference in temporal responsiveness.

# Reportable Outcomes

1. A manuscript detailing our identification of the source of low thresholds, the low axonal thresholds as well as the differences between ganglion cell types has been accepted and published.

2. A total of eleven presentations related to this work have been made or have been accepted at upcoming conferences: six were in the first year, four additional presentations were made this year. New presentations include three at the most recent ARVO conference (April 2008), one at the upcoming Bonn Conference on Retinal Prosthetics (Bonn, Germany) (September 2009) and one at the upcoming Military Health Forum in Kansas City (September 2009).

3. Findings from the work described here have been included as parts of two grant applications. The first is an R01 submitted in February 2009 (currently scored at 19$^{th}$ percentile and awaiting a funding decision). The second is a Merit Review application to the VA.

4. As the result of our collaboration with Alyosha Molnar, Assistant Professor in Electrical Engineering and Computer Science at Cornell University, we have developed a multi-compartment model with Hodgkin-Huxley kinetics. We have used the model to explore the role of the sodium channel band to electric stimulation (described above). A manuscript of this work is currently under development and we anticipate submission in fall 2009.

5. We are currently developing a publication on the temporal response properties that include results from Goal 3 (above) as well as some new findings not described in this write-up.

# Conclusions

To improve the clinical outcomes associated with retinal prosthetics, we are investigating the response of retinal neurons to electric stimulation. Our findings provide direct answers to several open questions in this area and provide guidance for future studies. Our continuing efforts will lead to new, more effective methods of stimulation that reduce the high threshold levels currently associated with clinical trials. Our findings will also form the basis for methods that improve the quality of elicited percepts.

We have determined that the site of lowest threshold in retinal ganglion cells (the output cells of the retina) is a small portion of the proximal axon, ~40 μm from the soma. This region of low threshold is coextensive with a dense band of sodium channels also localized within the proximal axon. The identification of this site, presumably the site of spike initiation, answers a fundamental question relating to electric stimulation of retinal neurons; it also refutes predictions from two earlier computational simulations each of which predicted a different site (soma and axon bend).

We found that the size and location of the sodium channel band was consistent within a given type of ganglion cell, however the band properties for each type were different. We are still studying the implications of this previously unreported finding; it is likely that the band differences influence the different spiking patterns generated normally by each type. There are several possible mechanisms by which this might occur however, and we are just beginning to investigate these different possibilities.

In addition to the role that the sodium channel bands play during normal light responses, band properties also influence the response to electric stimulation. We have found that the activation thresholds are different for different ganglion cell types; since our results here suggest that the sodium channel band is the source of the low thresholds, it seems reasonable to assume that differences in the band underlie the differences in threshold (e.g. long bands might result in low thresholds). Results from a computational model allow us to explore the effect of individual band parameters on threshold. They suggest that band length and the density of sodium channels within the band have the strongest influence. The model also suggests that the range of thresholds is limited by band properties to less than a factor of 10.

As we gain insight into the response differences between different neural types, we will start to be able to predict the neural activity underlying clinical responses. Ultimately, this will help guide the design of new stimulation systems that can selectively activate individual ganglion cell types. This will help elicit neural activity that more closely resembles the normal, physiological responses.

Knowledge of the site of spike initiation also allows us to more effectively explore the mechanism(s) underlying activation. For example, we are investigating how individual properties of the electric field influence spike initiation; knowledge of the spike

initiation site makes the investigation much more straightforward. Also, we can more clearly determine whether other neuronal elements (e.g. soma size) also contribute to threshold. These findings will contribute to a fundamental knowledge basis from which we can more rationally investigate the retinal response underlying clinical percepts. Ultimately, this knowledge basis will lead to improved methods of stimulation.

Each type of ganglion cell is capable of responding at frequencies > 200 Hz and many can respond at frequencies > 300 Hz. At least one type (the ON DS cell) can respond to frequencies > 500Hz. Presumably, one or more band properties influence these responses as well and we will explore this relationship in the upcoming year.

# References


1. Fried, S.I., H.A. Hsueh, and F. Werblin, *A method for generating precise temporal patterns of activity with prosthetic stimulation*, in *Artificial Sight: Basic Research, Biomedical Engineering and Clinical Advances*, M. Humayun, et al., Editors. 2007, Springer. p. 347-54.
2. Jensen, R.J., O.R. Ziv, and J.F. Rizzo, *Responses of rabbit retinal ganglion cells to electrical stimulation with an epiretinal electrode.* J Neural Eng, 2005. **2**(1): p. S16-21.
3. Sekirnjak, C., et al., *Electrical stimulation of mammalian retinal ganglion cells with multielectrode arrays.* J Neurophysiol, 2006. **95**(6): p. 3311-27.
4. Ahuja, A.K., et al., *An In Vitro Model of a Retinal Prosthesis.* IEEE Trans Biomed Eng, 2008. **55**(6): p. 1744-53.
5. Humayun, M.S., et al., *Pattern electrical stimulation of the human retina.* Vision Res, 1999. **39**(15): p. 2569-76.
6. Humayun, M.S., et al., *Visual perception in a blind subject with a chronic microelectronic retinal prosthesis.* Vision Res, 2003. **43**(24): p. 2573-81.
7. Rizzo, J.F., 3rd, et al., *Perceptual efficacy of electrical stimulation of human retina with a microelectrode array during short-term surgical trials.* Invest Ophthalmol Vis Sci, 2003. **44**(12): p. 5362-9.
8. Rizzo, J.F., 3rd, et al., *Methods and perceptual thresholds for short-term electrical stimulation of human retina with microelectrode arrays.* Invest Ophthalmol Vis Sci, 2003. **44**(12): p. 5355-61.
9. Zrenner, E., et al., *Subretinal chronic multielectrode arrays implanted in blind patients.* Invest Ophthalmol Vis Sci, 2006. **47**(1538).
10. Fried, S.I., et al., *Axonal sodium-channel bands shape the response to electric stimulation in retinal ganglion cells.* J Neurophysiol, 2009. **101**(4): p. 1972-87.
11. Schiefer, M.A. and W.M. Grill, *Sites of neuronal excitation by epiretinal electrical stimulation.* IEEE Trans Neural Syst Rehabil Eng, 2006. **14**(1): p. 5-13.
12. Greenberg, R.J., et al., *A computational model of electrical stimulation of the retinal ganglion cell.* IEEE Trans Biomed Eng, 1999. **46**(5): p. 505-14.
13. Jensen, R.J., et al., *Thresholds for activation of rabbit retinal ganglion cells with an ultrafine, extracellular microelectrode.* Invest Ophthalmol Vis Sci, 2003. **44**(8): p. 3533-43.
14. Sekirnjak, C., et al., *High-resolution electrical stimulation of primate retina for epiretinal implant design.* J Neurosci, 2008. **28**(17): p. 4446-56.
15. Fried, S.I., H.A. Hsueh, and F.S. Werblin, *A method for generating precise temporal patterns of retinal spiking using prosthetic stimulation.* J Neurophysiol, 2006. **95**(2): p. 970-8.
16. Rasband, M.N., et al., *Dependence of nodal sodium channel clustering on paranodal axoglial contact in the developing CNS.* J Neurosci, 1999. **19**(17): p. 7516-28.
17. Kordeli, E., S. Lambert, and V. Bennett, *AnkyrinG. A new ankyrin gene with neural-specific isoforms localized at the axonal initial segment and node of Ranvier.* J Biol Chem, 1995. **270**(5): p. 2352-9.

18. Jenkins, S.M. and V. Bennett, *Ankyrin-G coordinates assembly of the spectrin-based membrane skeleton, voltage-gated sodium channels, and L1 CAMs at Purkinje neuron initial segments.* J Cell Biol, 2001. **155**(5): p. 739-46.
19. Boiko, T., et al., *Functional specialization of the axon initial segment by isoform-specific sodium channel targeting.* J Neurosci, 2003. **23**(6): p. 2306-13.
20. Wollner, D.A. and W.A. Catterall, *Localization of sodium channels in axon hillocks and initial segments of retinal ganglion cells.* Proc Natl Acad Sci U S A, 1986. **83**(21): p. 8424-8.
21. Van Wart, A., J.S. Trimmer, and G. Matthews, *Polarized distribution of ion channels within microdomains of the axon initial segment.* J Comp Neurol, 2007. **500**(2): p. 339-52.
22. O'Brien, B.J., et al., *Intrinsic physiological properties of cat retinal ganglion cells.* J Physiol, 2002. **538**(Pt 3): p. 787-802.
23. Roska, B. and F. Werblin, *Vertical interactions across ten parallel, stacked representations in the mammalian retina.* Nature, 2001. **410**(6828): p. 583-587.
24. Hines, M., *NEURON - a program for simulaiton of nerve equations*. Neural Systems: Analysis and Modeling, ed. F. Eckman. 1993, Norwell: Kluwer Academic. 127-136.
25. Oesch, N., T. Euler, and W.R. Taylor, *Direction-selective dendritic action potentials in rabbit retina.* Neuron, 2005. **47**(5): p. 739-50.
26. Velte, T.J. and R.H. Masland, *Action potentials in the dendrites of retinal ganglion cells.* J Neurophysiol, 1999. **81**(3): p. 1412-7.
27. Carras, P.L., P.A. Coleman, and R.F. Miller, *Site of action potential initiation in amphibian retinal ganglion cells.* J Neurophysiol, 1992. **67**(2): p. 292-304.